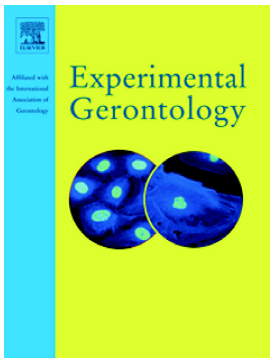

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PROTEOMIC ANALYSIS OF AGE-RELATED CHANGES IN OVINE CEREBROSPINAL FLUID

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ABSTRACT

Cerebrospinal fluid (CSF) circulates through the brain and has a unique composition reflecting the biological processes of the brain. Identifying ageing CSF biomarkers can aid in understanding the ageing process and interpreting CSF protein changes in neurodegenerative diseases. In this study, ovine CSF proteins from young (1-2 year old), middle aged (3-6 year old) and old (7-10 year old) sheep were systemically studied. CSF proteins were labelled with iTRAQ tagging reagents and fractionated by 2-dimensional high performance, liquid chromatography. Tryptic peptides were identified using MS/MS fragmentation ions for sequencing and quantified from iTRAQ reporter ion intensities at m/z 114, 115, 116 and 117. Two hundred thirty one peptides were detected, from which 143 proteins were identified. There were 52 proteins with >25% increase in concentrations in the old sheep compared to the young. 33 of them increased >25% but <50%, 13 increased >50% but <1 fold, 6 increased >1 fold [i.e. haptoglobin (Hp), haemoglobin, neuroendocrine protein 7B2, IgM, fibrous sheath interacting protein 1, vimentin]. There were 18 proteins with >25% decrease in concentrations in the old sheep compared to the young. 17 of them decreased >25% but <50%, and histone deacetylase 7 (HDAC7) was gradually decreased for over 80%. Glutathione S-transferase was decreased in middle aged CSF compared to both young and old CSF. The differential expressions of 3 proteins (Hp, neuroendocrine protein 7B2, IgM) were confirmed by immunoassays. These data expand our current knowledge regarding ovine CSF proteins, supply the necessary information to understand the ageing process in the brain and provide a basis for diagnosis of neurodegenerative diseases.

Highlights

- CSF protein changes during normal ageing process
- Seventy proteins were changed in concentration among different aged sheep CSF
- A common ageing CSF biomarker identified to date is immunoproteins
- Identifying ageing CSF biomarker help understand the ageing process in the brain

Key words: ageing, CSF, biomarkers, proteomics, choroid plexus, neurodegenerative disease

1. INTRODUCTION

Cerebrospinal fluid (CSF) is a clear, colourless bodily fluid that circulates through the brain and communicates freely with the brain extracellular fluid. In adult humans, about two-thirds of CSF is secreted by the choroid plexus (CP) at the rate of about 0.35-0.4 ml / min or 500-600 ml / day, while the remainder is from the extrachoroidal sources [1]. CSF has a unique composition that is different from the plasma, due to the presence of blood-brain barrier (BBB) and blood-CSF barrier (BCSFB), the active secretion at the CP and specific transport systems for moving substances between blood and CSF. In comparison to plasma ultrafiltrate, CSF contains higher concentrations of sodium, chloride, and magnesium and lower concentrations of glucose, proteins, amino acids, uric acid, potassium, bicarbonate, calcium and phosphate [2]. The CSF is in direct contact with the brain interstitial fluid, and the composition of the CSF therefore reflects biological processes occurring in the brain [3]. This understanding has led to an interest in the potential for discovering biomarkers within the CSF, which can be used to monitor brain function and aid in diagnosis of neurological diseases.

During ageing, several structural, chemical, and functional changes occur in the brain. The weight and volume of both cerebral cortex and hippocampus reduce, and the ventricular system expands to fill the space vacated by the brain parenchyma [4]. In contrast, the CP in the lateral ventricles doubles in weight, leading to significant increase of the ratio of the CP and the brain in weight [5,6]. The CSF secretion by the CP is decreased, leading to a decreased overall turnover of CSF which can affect its protein composition.

There are few studies on the CSF biology during ageing in absence of any neurological diseases, and the drastically incomplete knowledge hinders us from understanding the ageing process in the brain. In this study, we systemically characterized age-related protein changes in ovine CSF using a gel free proteomic mass spectrometry (MS) approach with isobaric labelled samples (iTRAQ) techniques. The advantage of using sheep in this study is that adequate CSF samples of all age groups can be obtained, with facile control over gender selection and environmental factors. Furthermore, we have previous characterized the age-related changes in the CP structure and function in the sheep, and found that the secretion rate of the CP is decreased thus slowed overall CSF turnover, which contributed to the increased protein concentrations within the CSF with increasing age [6-10].

2. METHODS

2.1. Sample collection

Clun Forest strain adult female sheep aged between 1 and 10 year old were divided into 3 groups: young (aged 1-2 years); middle-aged (3-6 years) and old (7-10 years). Each group comprises 7 sheep. Sheep were anaesthetized with *i.v.* thiopentone sodium (20 mg.kg^{-1}) and heparinised (20,000 IU heparin kg^{-1}). CSF samples were collected from the cisterna magna by needle puncture [11]. Samples were spun at 10 000 g for 10 min at 4 °C. CSF samples with any blood contamination determined by the presence of erythrocytes in the solution or precipitation were discarded. Supernatants were immediately stored at -80 °C until being analysed. All procedures were within the Home Office Scientific procedures Act, 1986 (HMSO, London, UK), and were approved by King's College London research ethics committee.

2.2. Sample preparation for MS analysis

Equal volumes of CSF samples from seven individuals within each group were pooled to give a final volume of 80 μl per age group. An internal standard (IS) was prepared by pooling equal amount of each sample from the analysis set. The pooled samples were then digested with sequencing grade porcine trypsin (Promega, Fitchburg, Wisconsin, USA) overnight at 37 °C, followed by reduction and alkylation steps performed according to the instructions outlined in the iTRAQ labelling kit (AB Sciex, Framingham, MA, USA). Following this, the digests were then dried down in a vacuum centrifuge and iTRAQ labelling carried out also according to instructions in the iTRAQ labelling kit. Each iTRAQ tag was assigned as follows: iTRAQ reagent 114 for Young, 115 for Middle-age, 116 for Old and 117 for the IS.

Both MS and MS/MS analysis was performed on the fractionated peptides using an Applied Biosystems 4800 MALDI TOF/TOF mass spectrometer. The mass spectrometer was operated under control of 4000 Series Explorer v3.5.2 software (Applied Biosystems, Waltham, Massachusetts, USA). A total of 1000 shots per MS spectrum (no stop conditions) and 2500 shots per MS/MS spectrum (no stop conditions) were acquired. The following MS/MS acquisition settings were used: 2KV operating mode with CID on and precursor mass window resolution set to 300.00 (FWHM). Peak lists of MS and MS/MS spectra were generated using 4000 Series Explorer v3.5.2 software and the following parameters were used after selective labelling of monoisotopic mass peaks: MS peak lists: S/N threshold 10, Savitzky Golay smoothing (3 points across peak (FWHM)), no baseline correction, MS/MS peak lists: S/N threshold 14; smoothing algorithm: Savitzky Golay, smoothing (7 points across peak (FWHM)).

2.3. Quantitative MS analysis

Liquid chromatography fractionation and subsequent MS analysis was conducted as described before [12]. Briefly, labelled tryptic peptides obtained from protein digestion were pooled and dissolved in 2.4 ml of SCX buffer A (10 mM phosphate, 20% acetonitrile) and centrifuged at 15,000 × *g* for 5 min to remove any insoluble debris. The peptide mixture was loaded onto a Polysulphoethyl A column (4.6 mm × 100 mm, 5 µm, 300 Å, PolyLC Inc.) using a flow rate of 800 µl / min. The bound sample was washed using SCX buffer A for approximately 20 min, until non-binding mixture components were removed (as determined by the UV traces returning to baseline levels). Peptides were then eluted at 400 µl / min using a gradient of SCX

buffer B (SCX A containing 1 M potassium chloride) of 0–30% over 30 min. Fractions were collected manually at 60 s intervals. SCX fractions containing peptides were dried to completeness and dissolved in 30 µl of RP buffer A (2% acetonitrile) before separating further using an Ultimate 3000 chromatography system connected to a Probot fraction collector (Dionex, Sunnyvale, CA, USA). Samples were randomised and loaded onto a C18 trapping column before eluting onto a 75 µm internal diameter C18 PepMap column. Peptides were washed for 15 min in RP buffer A before eluting with a 2–50% gradient of acetonitrile over 120 min, followed by further elution with 90% acetonitrile for 15 min. Eluted peptide fractions were spotted onto a MALDI-MS target plate every 10 s with 3 mg/ml α-cyano-2-hydroxy cinnamic acid at a flow rate of 1.2 µl/min. A blank injection was performed between peptide runs to minimise sample carry-over.

Peptides were analysed using an Applied Biosystems 4800 Proteomics Analyser with close external standards to calibrate the instrument. Survey spectra were collected from the range 900 to 4000 Da, with a focus mass of 2500 Da. Fifty shots were fired per sub-spectrum, with 1000 total shots per spectrum without stop conditions. All sub-spectra were accepted, and laser intensity was set to 3750. For product ion analysis, a maximum of 10 precursor ions per spot were selected automatically with a minimum signal-to-noise ratio of 40. Lowest-intensity precursors were acquired first.

An automated database search was run using GPS Explorer v3.6 (AB Sciex, Framingham, MA, USA). MASCOT was used as the search engine to search the NCBI non-redundant database version Oct 04, 2011, using the following search parameters: species = mammals; precursor ion mass tolerance of 150 ppm; MS/MS

fragment ion mass tolerance of 0.3 Da; iTRAQ fragment ion mass tolerance of 0.2 Da. Oxidation of methionine residues were allowed as variable modifications, and N-term (iTRAQ), lysine (iTRAQ) and MMTS modification of cysteine residues were set as fixed modifications. Protein were identified on the basis of having at least one peptide with an ion score above 95% confidence. All identified CSF proteins were subjected to functional analyses using PubMed search (https://www.ncbi.nlm.nih.gov/pubmed/) and the PANTHER classification system (http://www.pantherdb.org/genes/batchIdSearch.jsp) [13]. Only peptides unique for a given protein was considered for relative quantification. iTRAQ Ratios were normalized using the following formula: iTRAQ Ratio = Ratio/(median iTRAQ Ratio of all found pairs) that was applied in GPS Explorer software.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Experiments were performed using commercially available ovine protein ELISA kits: sheep haptoglobin (HP), sheep neuroendocrine protein 7B2 (MyBioscience, San Diego, CA, USA), according to the manufactory instructions. The target protein concentrations in CSF were determined according to the standard curve with known concentrations of the target proteins.

2.5. Western Immunoblotting

CSF samples (15 μl) were mixed with equal amount of 2x Laemmli sample buffer (Sigma-Aldrich, St. Louis, Missouri, USA). The samples were loaded onto precast 10% SDS–polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA) and were electrophoresed at 60 mV constant voltage until the dye front reached the bottom of the gel. The electrophoresed protein was transferred onto a 0.45 μm thick

nitrocellulose membrane, which was saturated for 1 h with 5% (w/v) milk powder in tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T). Membranes were then incubated with the primary antibodies rabbit anti-sheep mu chain (1:500)(Sigma-Aldrich, St. Louis, Missouri, USA), anti-sheep IgG (1:500)(Zymed-Thermo Fisher Scientific, Waltham, Massachusetts, USA); or anti-human haemoglobin β/γ/δ (H-76) (1:200)(Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. Thereafter the membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000)(Dako-Agilent technologies, Santa Clara, California, USA). After final wash, the immunostained proteins were developed using a Pierce ECL developing kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and were detected by a Fluorchem M image system (ProteinSimple, San Jose, California, USA). A CSF sample was used as an internal standard for each Western blotting experiment. The Western bolts images were analysed using Image studio lite version 5.2 software (LI-COR Biosciences, Lincoln, Nebraska, USA). Each band was analysed by manually selecting them to obtain the density measurement. The ratios of the sample density to the internal reference were used and compared among age groups.

2.6. Data Analysis

All values were expressed as mean ± SEM. One way ANOVA with Tukey post-hoc study was used to analyse comparisons among the 3 age groups. SPSS version 19 (SPSS INC., Chicago, USA) was used for the analysis. Values of $P < 0.05$ were considered statistically significant, and values of $P < 0.01$ were considered statistically highly significant.

3. RESULTS

3.1. Identification of proteins in CSF

The total protein concentration in the CSF increased with advancing age, as these sheep showed age-related reduction in CSF secretion / turnover [10]. We therefore applied equal amount volume of CSF in the analysis instead of using equal amount of CSF proteins. Two hundred and thirty one peptides were detected from the pooled CSF samples. All but 5 of the peptides were named. From these peptides, 143 proteins were identified. Of these proteins, 78 were identified with at least two peptides while the reminder was identified with a single peptide (Supplement 1 and 2). These proteins included not only relatively abundant proteins, such as albumin, IgG, transthyretin, transferrin, haptoglobin, complements, and various apolipoproteins, but also lower abundance peptides such as neuropeptide Y (see Supplement 1 and 2). The functional classification of the 143 identified proteins was listed in Table 1.

3.2. Quantitative analysis of CSF proteins in different age groups

iTRAQ not only identifies CSF proteins but also enable us to quantitatively compare the proteins with different labels [14]. Although the protein profile in each iTRAQ experiment may vary when complex protein mixture are analysed, quantitative comparison is valid when the same peptide is detected in samples at the same time. We therefore compared the ratios of an individual age group to the pooled CSF group. Our results demonstrated that there were substantial quantitative differences in many proteins between the three groups. We separated these proteins into 2 main groups: whose levels have increased (Table 2) or decreased (Table 3) greater than 25%. There were 52 proteins with >25% increase in concentrations in the old sheep compared to the young (Table 2). 33 of them increased >25% but <50% [e.g. insulin like growth

factor binding proteins (IGFBPs), Complements], 13 increased >50% but <1 fold [e.g. transthyretin (TTR), apolipoprotein C-III), 6 increased >1 fold [i.e. haptoglobin (Hp), haemoglobin (HGB), neuroendocrine protein 7B2, immunoglobin M (IgM), fibrous sheath interacting protein 1, vimentin] (Table 2). There were 18 proteins with >25% decrease in concentrations in the old sheep compared to the young. 17 of them decreased >25% but <50% (e.g. fibrinogen, transferrin), and histone deacetylase 7 (HDAC7) was gradually decreased for over 80% (Table 3). Glutathione S-transferase was decreased in middle aged CSF compared to both young and old CSF.

3.3. Validation of proteins associated with ageing process

To confirm peptide-based protein identifications and variant proteins as suggested by tandem MS, ELISA and Western Immunoblotting were performed on the CSF samples. As the first step towards verifying these ageing biomarkers in Table 2, that displayed more than 1 fold among the 3 age groups, all commercially available antibodies and ELISA kits for the sheep proteins were purchased for further analysis. These were sheep Hp ELISA kit, sheep neuroendocrine protein 7B2 ELISA kit, anti-sheep mu chain and sheep IgG antibodies. In addition, an antibody for human haemoglobin β/γ/δ (H-76) was bought as there were no specific antibodies for sheep haemoglobin. Specific ovine protein ELISA kits indicated there were significant increase of Hp and neuroendocrine protein 7B2 in the old CSF (Fig 1). Western blots of sheep mu chain showed significant increase in IgM in old CSF, in contrast, there were no changes in IgG among age groups (Fig 2). There were no bands on Western immunoblots with the anti-human haemoglobin β/γ/δ antibody on the ovine CSF samples (data not shown).

4. DISCUSSION

In this study, we studied ovine CSF proteome and compared CSF protein levels between different ages. We identified 143 different ovine CSF proteins using MS/MS fragmentation ions for sequencing, and generated the most complete characterization of ovine CSF proteome to date according to our knowledge. The number of proteins detected in ovine CSF was similar with in bovine CSF [15], but was lower compared to human CSF proteome [16], probably because both the ovine and bovine protein database are far less than completed than the human [17]. A large percentage of ovine CSF proteins (21.7%) are binding proteins, including the major CNS apolipoproteins (e.g. A, C, D, E), consistent with CSF "sink action" [1]. We did not find the apolipoprotein B, the major apolipoprotein in the blood that is not present in CSF, indicating the minimal blood contamination in our CSF samples [18,19]. To maximize efficient use of the MS, 7 ovine CSF samples with same age were pooled into a group, similar to our previous studies [12]. This approach will ensure adequate materials to identify low abundant proteins and minimized inter-subject variability, which was kept in minimum as the sheep was carefully controlled over their age, gender, disease states, season *etc*. The variability can be further addressed by performing careful follow-on / validation of aliquot from each individual animal.

Using iTRAQ reporter ion intensities at m/z114, 115, 116, 117, 52 proteins were found to be increased for more than 25% between ages, and 6 of them (neuroendrocrine protein 7B2, IgM, fibrous sheath interacting protein 1, Hp, HGB, vimentin) were gradually increased for more than one fold with age. There were 18 proteins with >25% decrease in concentrations between ages, and the HDAC7

gradually decreased in concentrations for more than 80%. GST decreased in middle aged CSF samples compared to both young and old CSF samples.

While high throughput proteomic analysis of CSF proteins selects candidate proteins for further study, candidate proteins identified by this approach need to be validated before their biological roles are pursued extensively. As the first step towards developing candidate ageing markers, we sought to use commercially available antibodies against the candidate proteins on individual ovine CSF samples. We used as much as possible of commercially available sheep protein diagnosis kits/antibodies, and validated 3 candidate proteins: Hp, neuroendocrine secretory protein 7B2 and IgM.

Hp is an acute-phase protein that scavenges HGB in the event of haemolysis, but also can be induced by inflammation [20, 21]. Human CSF Hp has a role in the protection of the CNS against autoimmune inflammatory responses after aneurysmal subarachnoid haemorrhage [22]. Increased human CSF Hp concentration were found in a number of neurological diseases, e.g. idiopathic normal pressure hydrocephalus, traumatic brain injury, Gullain-Barre syndrome and neuromyelitis optica [23, 24]. Chamoun et al. [25] reported there was an increased likelihood of detecting Hp in human CSF with age and suggested Hp was a marker of BCSFB dysfunction.

Neuroendocrine secretory protein 7B2 resides in the secretory granules of neuroendocrine cells and functions as a specific chaperone for the proprotein convertase 2 [26] as well as an anti-aggregation secretory chaperone associated with neurodegenerative diseases [27]. The normal concentration of 7B2 in human CSF is

10-100 folds greater than in plasma [26, 28], suggesting 7B2 is originated from the brain.

The brain has historically been considered an 'immune privileged region', as it is separated from peripheral circulation by BBB and BCSFB. However, the brain also contains microglia, the counterpart to macrophages which actively surveys the brain [29]. A recent study suggests that brain immune surveillance communicates with the immune system and can generate adaptive immune responses [30]. In our study, 13.3% identified CSF proteins were immunoproteins, which are proteins with immunological activities, e.g. immunoglubins and complements. Among them, IgM significant was increased in the old ovine CSF. IgM plays an important role in cytolytic reactions and agglutination [31], and is strongly related with a fatal neurodegenerative disease called prion disease [32]. There was a correlation between CSF IgM and CSF apolipoproteins in patients with neurological diseases [33]. In contrast, IgG levels in the CSF were not different between different age groups. IgM is the largest antibody and moves out of the brain only with CSF clearance, while IgG is the most abundant but the smallest antibody and moves out of the brain via additional efflux system [34]. The increase of IgM in the old CSF could be caused by either the old sheep were subjected to some infections that the young sheep were not, and / or the old sheep had slower CSF turnover rates compared to the young ones - [10].

The HGB expression in vertebrates was previously thought to be restricted to red blood cells, however the expression of HGB was found in both rat and human neurons [35, 36] and HGB was detected in the CSF of normal human controls [37]. HGB was

involved in neuronal mitochondrial energetics with epigenetic changes to histones and may provide neuroprotection in multiple sclerosis by supporting neuronal metabolism [38]. Although the sequence homology of haemoglobin β in *ovis aries* vs. human is 83% homology, Western blots with an anti-human haemoglobin β/γ/δ (H-76) antibody showed no reaction with the ovine CSF samples.

To our best knowledge, there are only 2 studies on ageing CSF biomarkers in human. Utilising a shotgun proteomics strategy in conjunction with ICAT (isotope-coded affinity tags), Zhang *et al.* [18] identified 312 different CSF proteins, of which 187 were identified with at least two peptides while the reminder was identified with a single peptide. The authors discovered 6 proteins that were changed more than one fold between the young and old human CSF, i.e. agrin, bromodomain, splice isoform γ-2 of P36873, protein phosphatase, hypothetical protein, serine/threonine protein phosphatase, ubiquinol-cytochrome *c* reductase. An important issue in Zhang's study is that the changes in the relative amount of proteins were measured with pooled CSF samples. The variability between humans is more than between sheep. The study could not define if the age-related difference found were due to difference in some individuals or being distributed over all participants. Although follow up immunoassays can partially address this problem, this approach is limited by availability of sufficiently sensitive antibodies. Another study on age-related changes in human CSF proteome was conducted by Baird et al. [19] using the Slow off-rate modified Aptamer Array technique. Baird et al. identified 82 proteins that were changed over age 28-82, most of which are associated with immune system activation or response to injury. These biomarkers overlap with our discovery in immunoproteins, indicating a role for inflammatory process in CNS ageing. A recent

study found in healthy ageing human CSF, there was a three-fold increase of soluble triggering receptor expressed on myeloid cell 2 (TREM 2), which functions in immune response and may be involved in chronic inflammation [39].

Ageing is associated with chronic inflammation. Inflammageing describes the close relationship between low-grade chronic inflammation and ageing in various organs, including the brain [40]. A number of theories have been developed to define the inflammageing, such as redox stress, mitochondrial damage, immunosenescence, endocrinosenescence, epigenetic modification etc [41]. The age-related changes in the brain including the inflammageing may provide a substrate for developing neurodegenerative disease. Indeed, inflammation has long been associated with neurodegenerative diseases [42]. Future studies on neurodegenerative diseases should consider the ageing factor. An important precondition for discovering CSF biomarkers in neurodegenerative diseases is an understanding of the dynamic nature of the CSF proteome during normal ageing process.

A number of Alzheimer's disease (AD) biomarkers, such as amyloid beta, tau, neurofilament light, were detected in ovine CSF. Total tau and neurofilament light levels were comparable with human levels [43]. We also found a few of AD related proteins in the ovine CSF, e.g. Apolipoprotein E, Amyloid beta A4 protein isoform b precursor, amyloid precursor-like protein 1, and they were not significantly different in concentrations among the age groups. Sheep are not known to develop AD naturally, probably due to their shorter lifespan compared to humans and being culled once past their useful lifespan for the farmers [43].

In conclusion, this study has generated detail knowledge of the ovine proteome and presented a number of suitable candidate proteins for further study in terms of their physiological variation in CSF during ageing. It would be important to validate the results of this study in CSF from humans in future and to conduct further experiments to understand their roles in healthy ageing and neurodegerative diseases. Changes in the CSF proteins during ageing without disease may be small and heterogeneous between individuals. Thus a combination of a number of studies on CSF ageing biomarkers would provide a full picture of ageing related changes in CSF proteome. A common ageing CSF biomarker identified to date is immunoproteins indicating a role of the immune response and inflammation in normal ageing process in the brain. MS analysis usually serves to identify candidates, functional and follow up studies will be needed for understanding the physiology of brain ageing as well as for recognizing neurodegenerative disease-associated changes in CSF proteome patterns.

COMPETING FINANCIAL INTERESTS

There is no conflict of interest.

AUTHOR CONTRIBUTIONS

This project was conceived by R.C. and J.E.P. The *in vivo* experiments in this paper were performed by C.P.C. and R.C., while the quantitative mass spectrometry experiment was conducted by H.R.F. Data were analysed and interpreted by R.C., H.R.F., and S.Z. The manuscript was prepared by R.C., S.Z., H.R.F., C.P.C, and D.G.A.M. All authors have read and approved the final copy.

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Table 1. Functional classification of ovine CSF proteins

Protein function group	Protein number	Percentage (%)
Binding proteins	31	21.7
Peptide hormones	14	9.8
Enzymes for metabolism/signalling	36	25.2
Structural molecules	23	16.1
Immunoproteins	19	13.3
Coagulation proteins	9	6.3
Unknown	11	7.7
Total	143	100

Table 2: A list of proteins showing more than 25% age-related increase in ovine CSF

Protein name	Accession number	Peptide count	Average iTRAQ ratio young/IS	Average iTRAQ ratio middle-age/IS	Average iTRAQ ratio old/IS	% increase
Haptoglobin	gi|258499	3	0.57 [3]	0.51 [3]	1.56 [3]	Age-related increase >1 fold
	gi|998960	1	0.43 [1]	0.39 [1]	1.44 [1]	
Haemoglobin	gi|122686 beta subunit	3	0.49 [3]	1.06 [3]	1.90 [3]	
	gi|1787 alpha subunit	1	0.95 [1]	1.56 [1]	1.88 [1]	
Vimentin	gi|21757045	2	0.47 [2]	0.44 [2]	1.06 [2]	
IgM (Ig mu chain)	gi|165945	2	0.56 [2]	0.61 [2]	1.21 [2]	
Neuroendrocrine protein 7B2 isoform 1	gi|221139785	1	1.17 [1]	1.83 [1]	2.50 [1]	
Fibrous sheath interacting protein 1	gil28175039	1	0.23 [1]	0.34 [1]	0.78 [1]	
Transthyretin	gil57526651	7	0.63 [11]	0.73 [11]	1.09 [11]	Age-related increase >50% but <1 fold
Serpin A3-1	gil31340900	4	0.73 [5]	0.63 [5]	1.28 [5]	
Apolipoprotein C-III	gil47564119	3	0.74 [3]	1.09 [3]	1.22 [3]	
Inter-alpha-trypsin inhibitor	gil59857769	2	0.95 [2]	1.16 [2]	1.57 [2]	
	gil48374067	2	0.82 [2]	1.12 [2]	1.68 [2]	
Actin	gil929752	1	0.78 [1]	0.82 [1]	1.55 [1]	
Calcium uniporter channel	gil833995	1	1.28 [1]	0.88 [1]	2.36 [1]	
Thyroxine-binding globulin	gil155369640	1	0.66 [1]	0.62 [1]	1.07 [1]	
Beta-actin-like protein 2-like	gil57043600	1	0.78 [1]	0.82 [1]	1.55 [1]	
Retinoic acid receptor responder protein 2	gil346716116	1	0.58 [1]	0.97 [1]	0.91 [1]	
Neuropeptide Y	gil30923117	1	0.78 [1]	1.4 [1]	1.54 [1]	
Pyruvate kinase isozymes m1/m2 isoform	gil33286418	1	1.05 [1]	0.59 [1]	1.61 [1]	
Serum paroxonase/arylesterase 1	gil126722853	1	0.99 [1]	0.91 [1]	2.18 [1]	
Complement C1r-B	gil164518925	1	0.91 [1]	0.91 [1]	1.44 [1]	
Ceruloplasmin	gil57617174	14	0.85 [17]	0.84 [17]	1.15 [17]	Age-related increase >25% but <50%
Clusterin	gil27806907	7	0.81 [9]	0.93 [9]	1.10 [9]	
IGF-2	gil57164331	2	0.83 [2]	1.43 [2]	1.20 [2]	
IGFBP-2	gil57164237	7	0.90 [7]	1.14 [7]	1.25 [7]	
IGFBP-4	gil27807009	1	0.59 [1]	0.82 [1]	0.76 [1]	
IGFBP-5	gil263306	1	1.69 [1]	1.32 [1]	2.19 [1]	
IGFBP-6	gil56849568	1	0.39 [1]	0.72 [1]	0.82 [1]	
	gil5705897	1	0.79 [1]	1.13 [1]	0.96 [1]	
IGFBP-7	gil4504619	3	0.75 [3]	0.61 [3]	1.01 [3]	
Beta 2-microglubin	gil57164311	4	0.91 [4]	0.99 [4]	1.17 [4]	

Primary amine oxidase	gil30794300	3	0.75 [3]	0.91 [3]	1.06 [3]	
Complement C4	gil1227	7	0.84 [7]	0.96 [7]	1.06 [7]	
	gil1235	5	0.76 [5]	0.91 [5]	1.06 [5]	
	gil50567362	4	0.87 [4]	1.08 [4]	1.14 [4]	
	gil1314244	1	0.96 [1]	1.52 [1]	1.64 [1]	
Complement C3	gil4093220	1	0.76 [1]	0.96 [1]	1.03 [1]	
Complement C6	gil47115536	1	0.76 [1]	1.20 [1]	0.98 [1]	
Complement C8	gil841248	1	0.61 [1]	0.73 [1]	0.78 [1]	
Chromogranin B	gil12644006	2	1.00 [2]	1.28 [2]	1.32 [2]	
Alpha-macroglubin	gil290543549	2	0.78 [2]	0.92 [2]	1.17 [2]	
Beta-2 glycoprotein 1	gil54792721	2	0.79 [3]	1.01 [3]	1.06 [3]	
	gil57528174	2	0.65 [2]	0.81 [2]	1.14 [2]	
Plasminogen	gil833995	2	0.89 [2]	0.87 [2]	1.12 [2]	
	gil51702791	1	0.83 [1]	0.99 [1]	1.12 [1]	
Proactivator polypeptide	gil27806447	2	0.60 [2]	0.76 [2]	0.83 [2]	
Apolipoprotein D	gil348582766	1	0.71 [1]	0.99 [1]	0.90 [1]	
Brain-type ribonuclease	gil2381819	1	0.82 [1]	1.37 [1]	1.22 [1]	
Extracellular superoxide dismutase	gil130508829	1	0.54 [1]	0.85 [1]	0.72 [1]	
Phosphatidylcholine sterol acyltransferase	gil21542351	1	1.10 [1]	0.90 [1]	1.62 [1]	
Eukephalin	gil223387	1	0.86 [1]	1.07 [1]	1.10 [1]	
Neuroserpin	gil18158628	1	0.43 [1]	0.66 [1]	0.58 [1]	
Alpha-2 antiplasmin	gil27807209	1	0.58 [1]	0.72 [1]	0.82 [1]	
Niemann-pick C2 protein	gil28373999	1	0.74 [1]	0.71 [1]	0.94 [1]	
Rheumatoid factor Fab	gil3659940	1	1.07 [1]	1.42 [1]	1.60 [1]	
Neuroblastoma suppressor	gil13928832	1	1.04 [1]	1.07 [1]	1.35 [1]	
Malate dehydrogenase	gil5174539	1	0.75 [1]	0.72 [1]	0.99 [1]	
Alpha-1 antiproteinase	gil461443	1	0.28 [1]	0.28 [1]	0.40 [1]	
Kininogen-1	gil57109938	1	0.74 [1]	0.97 [1]	1.11 [1]	
Fibromodalin	gil453157	1	0.78 [1]	0.84 [1]	0.91 [1]	

Column headings refer to the following: accession number is the reference for each protein in the NCBInr database; peptide count refers to the number of unique peptides with MS/MS ion scores used for protein identification; the average iTRAQ ratios are shown after data normalisation followed by the number of peptides used for quantification in square brackets. IS = internal standard.

Table 3: A list of proteins showing more than 25% age-related decrease in ovine CSF

Protein name	Accession number	Peptide count	Average iTRAQ ratio young/IS	Average iTRAQ ratio middle-age/IS	Average iTRAQ ratio old/IS	% decrease
Histon deacetylase 7	gi|30913010	1	3.85 [1]	1.39 [1]	0.76 [1]	Age-related decrease >80%
Fibrinogen	gil1346006	3	1.25 [3]	1.40 [3]	0.74 [3]	Age-related decrease >25% but <50%
	gil3789962	3	1.03 [3]	1.15 [3]	0.87 [3]	
	gil6980816	2	1.23 [2]	1.42 [2]	0.92 [2]	
Neurosecretory protein VGF	gil17136078	2	1.39 [2]	1.04 [2]	0.99 [2]	
Beta-1,3-N-actyl glucosamingl transferase 6	gil61553937	2	1.64 [2]	1.38 [2]	1.17 [2]	
Tetranectin	gil37409	2	1.23 [2]	1.37 [2]	0.83 [2]	
Transferrin	gil2318026	2	1.40 [2]	1.09 [2]	0.98 [2]	
Calreticulin	gil545920	2	0.95 [2]	1.11 [2]	0.64 [2]	
	gil237420	1	0.91 [1]	0.99 [1]	0.55 [1]	
Contactin-2	gil4827022	2	1.25 [2]	0.73 [2]	0.77 [2]	
V-set and transmembrane domain protein 2A	gil20306326	1	1.12 [2]	0.81 [2]	0.59 [2]	
Cadherin-15	gil4826669	1	1.39 [1]	0.89 [1]	0.72 [1]	
Chain A, Divalent cation tolerant protein cuta	gil56554578	1	1.37 [1]	1.04 [1]	1.00 [1]	
Cell adhesion molecule 4	gil21686977	1	1.11 [1]	0.50 [1]	0.77 [1]	
Adipsin / complement factor D	gil773265	1	1.50 [1]	1.27 [1]	1.09 [1]	
Nidogen-2	gil2791962	1	1.35 [1]	1.22 [1]	0.70 [1]	
Protein C	gil163487	1	0.93 [1]	0.68 [1]	0.69 [1]	
Neurocan core protein	gil77681353	1	1.11 [1]	0.71 [1]	0.61 [1]	
Protein kinase C-binding protein NELL-2	gil2494290	1	1.81 [1]	1.40 [1]	1.00 [1]	
Nuclear factor of activated T cells cytoplasmic isoform 2	gil27886541	1	0.96 [1]	1.56 [1]	0.38 [1]	

Column headings refer to the following: accession number is the reference for each protein in the NCBInr database; peptide count refers to the number of unique peptides

with MS/MS ion scores used for protein identification; the average iTRAQ ratios are shown after data normalisation followed by the number of peptides used for quantification in square brackets. IS = internal standard.

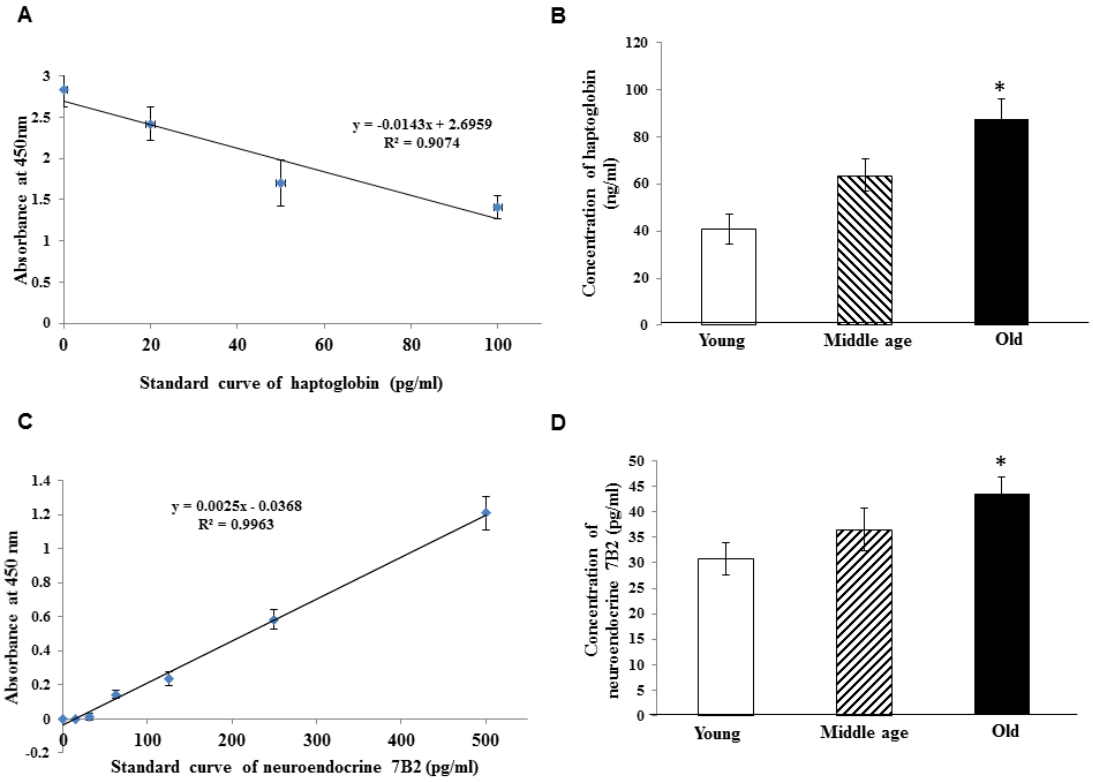

Figure 1. Concentration of haptoglobin (HP) and neuroendocrine protein 7B2 in CSF of young, middle age, and old groups by ELISA assay. A, C are standard curves of Hp (A) and neuroendocrine 7B2 (C); B and D summarized the concentrations of Hp (B) and neuroendocrine 7B2 (D) in CSF from different age groups, n = 7. *compared to young group, $P<0.01$.

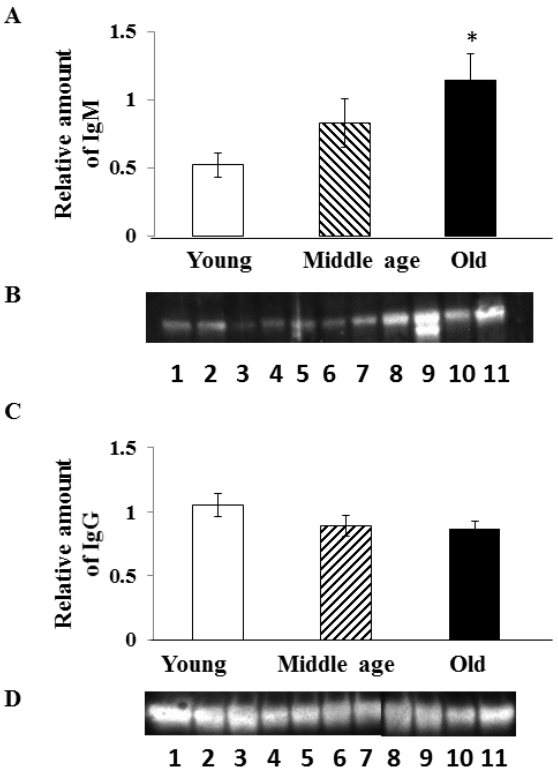

Figure 2. Relative amount of IgM (A) and IgG (C) in CSF between different ages, which was based on their band density of *Western* immunoblotting image of IgM (B) and IgG (D) respectively. In image B or D, lanes 1-4: young CSF; lanes 5-7: middle aged CSF; lanes 8-10: old CSF; lane 11: molecular size marker. *compared to young group, $P<0.01$